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A Dual Luciferase Multiplexed High-Throughput Screening Platform for Protein-Protein Interactions

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To study the biology of regulators of G-protein signaling (RGS) proteins and to facilitate the identification of small molecule modulators of RGS proteins, the authors recently developed an advanced yeast 2-hybrid (YTH) assay format for GαZ and RGS-Z1. Moreover, they describe the development of a multiplexed luciferase-based assay that has been successfully adapted to screen large numbers of small molecule modulators of protein-protein interactions. They generated and evaluated 2 different luciferase reporter gene systems for YTH interactions, a Gal4 responsive firefly luciferase reporter gene and a Gal4 responsive *Renilla* luciferase reporter gene. Both the firefly and *Renilla* luciferase reporter genes demonstrated a 40- to 50-fold increase in luminescence in strains expressing interacting YTH fusion proteins versus negative control strains. Because the firefly and *Renilla* luciferase proteins have different substrate specificity, the assays were multiplexed. The multiplexed luciferase-based YTH platform adds speed, sensitivity, simplicity, quantification, and efficiency to YTH high-throughput applications and therefore greatly facilitates the identification of small molecule modulators of protein-protein interactions as tools or potential leads for drug discovery efforts. (*Journal of Biomolecular Screening* 2003:676-684)

Key words: dual luciferase, high throughput, yeast, yeast 2-hybrid, RGS protein

INTRODUCTION

HETEROTRIMERIC G-PROTEINS play an important role in diverse cellular processes by transmitting signals from 7-transmembrane receptors to intracellular signaling pathways.[1,2] Although G-proteins consist of α and βγ subunits, they are classified by the identity of the guanine-binding α subunit, and to date, at least 20 human genes have been identified. Gα subunits are in an active state when bound to GTP and are inactivated when GTP is hydrolyzed by the Gα subunits' intrinsic GTPase activity.[3] Recently, a novel class of proteins has been identified that attenuates the activity of heterotrimeric G proteins.[4-10] These regulators of G protein signaling (RGS) proteins function by modulating the endogenous GTPase activity of the Gα subunits, thereby allowing the signal through G-proteins to turn off more rapidly (than endogenous GTPase activity) after removal of ligand and to inhibit background signaling.

To study the role of G-protein signaling and RGS modulation in the etiology of neuropsychiatric disorders such as depression and anxiety, we decided to focus our investigation on GαZ and RGS-Z1. GαZ is of particular interest due to a very limited expression pattern (mainly in the retina, brain, and adrenal medulla),[11] whereas many other Gα subunits are more ubiquitously expressed. In addition, GαZ is pertussis toxin insensitive,[11] which further distinguishes it from other Gα subunits. Expression of RGS-Z1, however, is limited to the brain, with a particular high level of expression in the caudate nucleus.[12,13] Furthermore, GαZ knock-out mice demonstrate decreased (antidepressant) effects of catecholamine reuptake inhibitors as well as an increased response to cocaine and a reduced analgesic effect of morphine.[14] A compound that is able to inhibit RGS-Z1 function is speculated to prolong GαZ-linked receptor function, which may, at least in part, be opposite to a GαZ knock-out. In addition, because RGS-Z1 can also enhance the endogenous GTPase activity of Gαi-1,[15] inhibitors to RGS are anticipated to have potential therapeutic utility in the treatment of depression and neuropathic pain.

To further study the biology of RGS proteins and identify potential small molecule tools that modulate RGS-Z1, a high-throughput screening (HTS) platform was developed based on the yeast 2-hybrid[16] (YTH) interaction between a constitutatively active GαZ(Q205L)[13] and RGS-Z1.[12] Previous YTH screening methods used an agar-based platform.[17] To develop a YTH-based assay format amenable for high throughput, we investigated luci-

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Received Jun 2, 2003, and in revised form Jul 18, 2003. Accepted for publication Jul 23, 2003.

Journal of Biomolecular Screening 8(6): 2003
DOI: 10.1177/1087057103258287
Published by Sage Publications in association with The Society for Biomolecular Screening

ferase reporter systems. Despite being commonly used as a reporter gene in mammalian cell-based systems,[18] luciferase (luc) has not been used in an analogous manner in yeast.[19] In addition to firefly (*P. pyralis*) luciferase,[20] many mammalian cell-based systems use the luciferase enzyme from the anthozoan coelenterate *Renilla reniformis* (sea pansy) as a control reporter.[21] The light produced by firefly luciferase involves the oxidation of beetle luciferin in a reaction requiring ATP and Mg^{2+}, whereas the light produced by *Renilla* luciferase involves the oxidation of coelenterazine, which is not dependent on ATP and Mg^{2+}. More importantly, this substrate specificity allowed the sequential measurement of both luciferase enzyme activities inside the same assay well (multiplexed assay).[22] Furthermore, the use of a luciferase-based YTH assay system enabled enhanced sensitivity and provided quantitative data over a large dynamic range, whereas the availability of luciferase substrates with a long light emission half-life enabled the adaptation to 384-well HTS platforms.

Here we describe the generation and evaluation of 2 luciferase reporter gene systems for YTH interactions, a Gal4 responsive firefly luciferase (FF-Luc) reporter gene and a Gal4 responsive *Renilla* luciferase (Ren-Luc) reporter gene. Both the firefly and *Renilla* luciferase reporter genes demonstrated a 40- to 50-fold increase in luminescence in strains expressing interacting YTH fusion proteins versus negative control strains. Standard YTH auxotrophic reporter genes such as His3 or Leu2 link positive protein-protein interactions to a change in yeast cell phenotype (growth) on selective media.[23] The YTH luciferase reporters described here significantly decreased assay time (hours vs. several days) as compared to standard auxotrophic reporters[23] and increased sensitivity as compared to commonly used YTH reporters. In addition, each luciferase single reporter assay was adaptable to HTS format, and 2 separate yeast strains containing either the firefly or the *Renilla* luciferase reporter gene could be multiplexed. The multiplex format enabled internal controls and assay efficiency for 2 screens to enable simultaneous identification and adaptation of small molecule modulators, as tools and potential drug discovery leads for multiple protein-protein interactions.

MATERIALS AND METHODS

Molecular reagents

Standard molecular biology techniques were used as described elsewhere.[24] PCR reagents were from BD Biosciences Clontech (Palo Alto, CA) and Stratagene (La Jolla, CA). Oligonucleotides were purchased from Invitrogen (Carlsbad, CA). The complete ORFs for GαZ and RGS-Z1 (Genbank accession numbers J03260 and AF074979, respectively) were cloned from a human brain cDNA library (quickclone cDNA, BD Biosciences Clontech, Palo Alto, CA) by PCR amplification. PCR primers were designed at the 5′ and 3′ ends of the open reading frame of GαZ and RGS-Z1. PCR amplification was performed under standard buffer conditions using a cDNA Advantage cDNA kit (BD Biosciences Clontech, Palo Alto, CA). The primers used were GαZ-fwd 5′-ATGGGATGTCGGCAAA GCT CAGAGGAAA-3′ and GαZ-rev 5′-CAAGGGGTGGGG GACATT-3′ and RGS-Z1-fwd 5′-CCCGGCCGGCAGGTGGAC-3′ and RGS-Z1-rev 5′-CTCATGCAAAATAAAAGTGGTTC-3′. Amplified DNA fragments corresponding to the expected product lengths for GαZ and RGS-Z1, respectively, were gel purified using GeneClean Spin columns (Q-Biogene, Carlsbad, CA). The gel-purified fragments were then TA-subcloned in the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). Inserts of several individual clones were confirmed by fluorescent dye terminating sequencing. The pCRII plasmid containing the entire wild-type ORF of GαZ was digested with *NcoI* and *EcoRI*. The insert containing the GαZ ORF was gel purified and ligated directionally in frame in the *NcoI* and *EcoRI* sites of the vectors pGBKT7 and pACT2 (BD Biosciences Clontech, Palo Alto, CA). The pCRII plasmid containing the ORF of RGS-Z1 was digested with *XhoI* and *BamHI*, blunt ended, and after gel purification, the insert was ligated in frame in the *SmaI* site of pGBKT7 and pACT2. The Q205L mutation in GαZ was generated in the pGBKT7 and pACT2 plasmids using the QuickChange kit (Stratagene, La Jolla, CA) and the primers 5′-GTGGGGGGGCTGAGGTCAGAG-3′ and 5′-CTCTGACCT CAGCCCCCCCAC-3′. Mutants were identified by sequence analysis of the resulting colonies.

YTH luciferase reporter vectors

A Gal4 firefly luciferase reporter plasmid (pEK1_FF-Luc) was described previously.[15] A Gal4 responsive *Renilla* luciferase reporter plasmid (pEK1_Ren-Luc) was created by release of the *R. reniformis* luciferase gene from pRL-null (Promega, Madison, WI), followed by a blunt-end ligation downstream of the Gal4 responsive promotor in pEK1 plasmid.[25] To improve the stability and half-life of the *Renilla* luciferase protein, the most 3′ cysteine residue was mutated to an alanine as described previously.[26]

Generation of YTH strains

Yeast transformations were carried out as described previously using the Li-acetate method and grown on synthetic complete drop-out media to maintain plasmids.[27] The activation domain and binding domain vectors containing GαZ(Q205L) and RGS-Z1 were transformed into yeast strain CY770[25] together with a ura-marked plasmid containing one of the luciferase reporter genes (firefly or *Renilla* luciferase). Similar strains expressing the potassium channel Kv4.3 (binding domain YTH vector) and its interacting protein KChIP1 (potassium channel interacting protein 1) (in the activation domain YTH vector) were constructed.[22] Transformants were grown for 3 to 4 days at 30 °C on SC -ura, -leu, -trp medium. Representative colonies of each transformation were tested for luciferase activity. Briefly, colonies were inoculated in 3 ml SC -ura, -leu, -trp medium and grown overnight at 30 °C. The

next morning, OD_{600} was measured, and culture densities were adjusted to an OD_{600} of 0.2. Cells were then seeded in 96-well plates (100 µl) and grown for an additional 3 h. Luciferase substrate (Promega, Madison, WI) was added (100 µl), and the plate was incubated for 60 min at RT while shaking. Luminescence was determined using a TopCount plate reader (Packard/Perkin Elmer, Wellesley, MA), followed by the addition of 100 µl *Renilla* luciferase substrate "Stop & Glow" (Promega, Madison, WI) and subsequent reading of luminescence.

High-throughput compound screening conditions

Test and control yeast strains were mixed in SC-leu-trp-ura medium and diluted to OD_{600} of 0.2. Then, 25 µl of diluted mixed yeast strains was added to compound containing 384-well plates (10 µl compound [dissolved in 100% DMSO] per well, 14 µg/ml final concentration). Yeast and compound were incubated for 3 h at RT, followed by the addition of 25 µl of firefly luciferase reagent (Promega, Madison, WI) to each well, and incubated for 1 h. Luminescence (firefly luciferase reporter) was determined using a Viewlux plate reader (Perkin Elmer, Wellesley, MA). After reading the firefly luciferase counts, using a Victor2 plate reader (Perkin Elmer, Wellesley, MA) equipped with an injection system and interfaced in a Thermo-CRS stacker-based robotic system (Thermo-CRS Ltd., Burlington, Ontario, Canada), 25 µl *Renilla* luciferase reagent (Stop & Glow, Promega, Madison, WI) was added to each well individually, and luminescence was determined immediately (within seconds, due to the short half-life of the *Renilla* luminescence). Approximately 26,800 compounds were assayed per day. Note that recently, a glow-type *Renilla* luciferase substrate has become available (Dual Glow Luciferase Assay System, Promega, Madison, WI), allowing more flexibility in HTS logistics. For example, immediate plate reads are not necessary with the glow-type substrate.

HTS data regression analysis

An SAS-based program[29] with an Excel interface was developed to calculate the means and standard errors for firefly (FF) and *Renilla* (RL) luciferase assay data and to plot the data by plate, row, and column. The wells at the edge of each plate can be deleted if a substantially different pattern is observed in comparison to other wells in the plate. Linear regression analysis was applied to the FF and RL data sets, respectively, which included 2 factors: plate and the time lag between filling a well with substrate and detection of luminescence. Because mechanical errors introduced either by photo-multiplier tube configurations or plate fill patterns resulted in some of the data sets to display a "zigzag" pattern in column data for both luciferase reporters (see the Results section), the regression analysis also contained an "odd or even" number column option. The FF residuals and RL residuals were then plotted. Compounds were selected for further testing based on residual values that fell within user-defined ranges of FF residual and RL residual values.

Single-dose dual luciferase data analysis

Each compound that was tested using the test and control strain in the established and the switched luciferase assay can be associated with 4 variables: control-FF, control-RL, test-FF, and test-RL. Each variable was first standardized (divided) by its plate mean, and then 2 new variables were defined as diff1 = (standardized control-FF) – (standardized test-RL) and diff2 = (standardized control-RL) – (standardized test-FF). After diff1 and diff2 values were plotted for each compound, 2 positive numbers, b and h, were intuitively chosen. The value of b and h determined the number of compounds that were tested in the subsequent dose-response assay (number of "positive" compounds). The number h defined the size of the 9 areas in the plot of diff1 and diff2 (see also Fig. 4E), whereas the value of b was used to define a 4-dimensional space containing compounds that were considered toxic. As a result, compounds were classified as follows: "toxic," if all 4 standardized variables were less than b; "no effect," if abs(diff1) < h and abs(diff2) < h; "positive," if diff1 > h and diff2 > h or diff1 < –h and diff2 < –h; "quencher," if diff1 > h and diff2 < –h, or diff1 < –h and diff2 > h; or "unknown" otherwise.

Dual luciferase dose-response assay data analysis

The data set was analyzed using 2-way layout ANOVA with the factors of group, dose, and Group × Dose interaction. FF-Luc and RL-Luc data was first compared at each dose level, and then the *p*-values were adjusted using the Sidak-Holm step-down method[29] to produce a comparison of test and control strain data over a given dose range.

Single turnover GTPase assay

The GTPase activity of GαZ and Gαi-1 proteins was determined by examining free γ-P_i release from bound GTP as described previously.[15] A truncated version of the human GαZ protein[11] corresponding to amino acids 24-350 was subcloned as a *NcoI – XhoI* fragment in pET16b vector (Novagen, Madison, WI). Inserts were verified by sequence analysis. Recombinant GαZ-6His protein was purified from *Escherichia coli* via standard Nickel-NTA resin-based chromatography. RGS-Z1, RGS4, and Gαi-1 recombinant protein were expressed and purified as described.[15]

RESULTS

Design of dual luciferase YTH assay

Previously, a YTH screen for small molecule inhibitors was conducted using an agar format.[17] To realize the speed and automa-

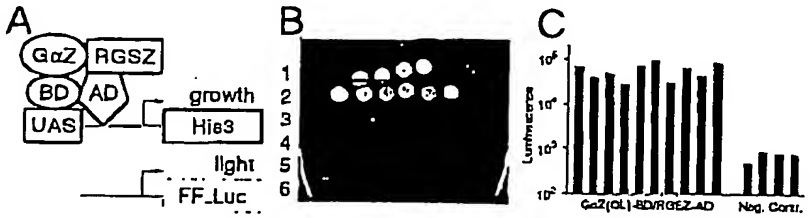

FIG. 1. The interaction between constitutively active GαZ(Q205L) and RGS-Z1. BD and AD indicate Gal4 DNA binding domain and activation domain fusion proteins, respectively. (A) His3 and FF-Luc represent yeast 2-hybrid (YTH) histidine and firefly luciferase reporter genes, respectively. (B) Histidine prototrophy was observed when GαZ(Q205L) was fused to the Gal4-BD and RGS-Z1 was fused to the Gal4-AD (boxed) but not when expressed as opposite fusion proteins. 1 = Kv4.3_BD/KChIP1_AD, 2 = GαZ(Q205L)_BD/RGS-Z1_AD, 3 = RGS-Z1_BD/GαZ(205L)_AD, 4 = empty_BD/GαZ(205L)_AD, 5 = RGS-Z1_BD/empty_AD, 6 = empty_BD/RGS-Z1_AD. (C) YTH luciferase reporter gene function. Individual isolates of GαZ(Q205L)/RGS-Z1 YTH interaction ($n = 10$) activated the firefly luciferase reporter gene (FF-Luc), resulting in a luminescence signal that is approximately 40-fold higher than in negative control strains (GαZ(Q205L)/empty vector) ($n = 6$).

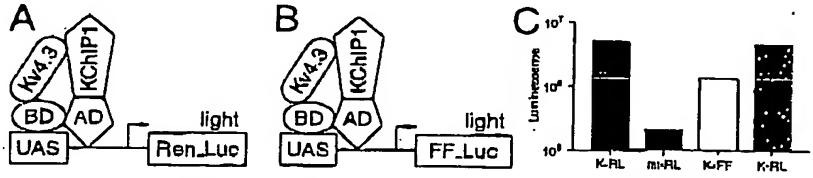

FIG. 2. Functionality of the *Renilla reniformis* (sea pansy) luciferase yeast 2-hybrid (YTH) reporter gene. The Kv4.3/KChIP YTH interaction also activated the *R. reniformis* luciferase YTH reporter gene (A, C: K-RL). A 40-fold induction of the *Renilla* luciferase reporter was obtained with the positive interaction pair (K-RL) versus negative controls (Kv4.3/empty vector, mt-RL graph B). The 2 Kv4.3/KChIP1 strains, each having one of the luciferase reporter genes (FF-Luc or Ren-Luc, A or B), were combined in a single well and assayed for reporter gene activity (K-FF and K-RL, open and gray bar in C).

tion necessary to aggressively pursue potential modulators of protein-protein interactions, YTH-based assays would benefit from a quantitative rapid reporter system that could be adapted into a liquid-based HTS assay format. Because the majority of the YTH reporters currently in use have an assay window of several days,[23] we developed and validated the functionality of several luciferase reporter genes in various YTH interactions. We developed the functionality of the luciferase reporters with 2 different YTH interactions, one being an interaction between a constitutively active GαZ(Q205L) and its regulator RGS-Z1 (referred to as the test strain), the other being an interaction between the potassium channel Kv4.3 and the interacting protein KChIP1[28] (control strain). The constitutively active GαZ(Q205L) interacts with RGS-Z1 (Fig. 1A) as is evident from positive growth on plates lacking histidine (Fig. 1B). Histidine auxotrophy was observed only when GαZ(Q205L) was fused to the Gal4-BD (BD = DNA binding domain) and RGS-Z1 was fused to the Gal4-AD (AD = DNA activation domain) and not when fused in the opposite orientation. This is most likely due to conformational differences. The Kv4.3/KChIP1 YTH interaction was also able to activate the His3 reporter gene (positive control).

The interacting YTH fusion proteins also activated a Gal4 responsive firefly reporter gene (FF-Luc). As is shown in Figure 1C, the GαZ(Q205L)/RGS-Z1 YTH interaction was able to activate the firefly luciferase reporter gene, resulting in a luminescence signal that was approximately 40-fold higher than in the negative control strains.

To broaden the utility of our YTH-luciferase assay, a 2nd luciferase reporter gene was developed and evaluated using *R. reniformis* luciferase downstream of the Gal-UAS (Ren-Luc) and tested in our YTH assays. A 50- to 100-fold induction of the *Renilla* luciferase reporter was obtained with the Kv4.3/KChIP1 interaction pair (Fig. 2A, 2C, "K-RL") versus negative controls ("mt-RL" in Fig. 2C). These results were similar to the results obtained with the firefly luciferase reporter (Fig. 2B). As is also shown in Figure 2C, 2 Kv4.3/KChIP1 strains having different luciferase reporter genes (firefly and *Renilla*) could be mixed in a single well and assayed sequentially for reporter gene activity using dual-luciferase assay reagents (K-FF and K-RL).

The established functionality of both firefly and *Renilla* YTH reporter genes allowed multiplexing YTH strains of interest. In the multiplexed YTH assay, 2 yeast strains (test and control) were mixed together within the same well for HTS. This enabled internal assay control, efficacy in assay implementation, conservation of materials, and increased information generated per assay. Because the luminescence output of the *Renilla* luciferase is substantially higher than that of firefly luciferase, the *Renilla* luciferase reporter gene was used for the less robust of the YTH interactions, GαZ(Q205L)/RGS-Z1. This interaction/reporter multiplex design enabled the most comparable counts within the assay format.

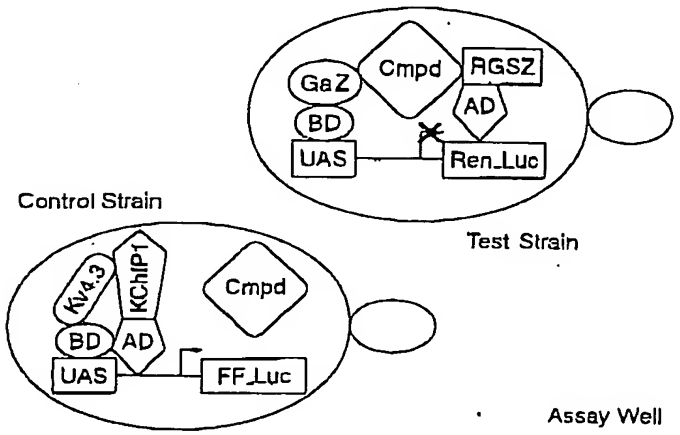

FIG. 3. The dual luciferase multiplexed high-throughput yeast 2-hybrid (YTH) assay. The test strain with the *Renilla* luciferase reporter (Ren-Luc) and the control strain with the firefly luciferase reporter (FF-Luc) were mixed within a single well for high-throughput screening. Compounds that were able to block the interaction between GαZ(Q205L) and RGS-Z1 negate reporter gene activity and were identified by a decrease in *Renilla* luciferase activity. The Kv4.3/KChIP interaction was not affected by this compound; the YTH interaction resulted in an increased firefly luciferase activity.

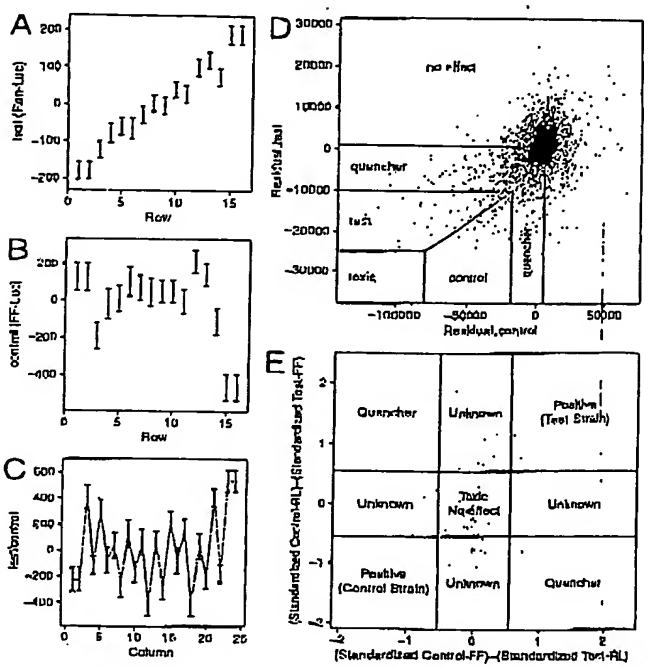

FIG. 4. Regression analysis of *Renilla* luciferase and firefly luciferase raw data from test and control strains identified automation-induced mechanical bias. Means and standard errors were calculated for test strain and control strain data and plotted by row and column. Samples in the outer wells of the plate exhibited a substantially different pattern from other wells in the plate (A, B, C). Analysis of test data by row (*Renilla* reporter) identified a drift in baseline (A). A typical up-and-down movement for both test and control strain was detectable in data analyzed by column (both test and control strain, C). Plot of residual values for both test and control strain (D) was used to select compounds for additional testing. Single-dose assay data were analyzed and plotted (E) by a custom SAS-based Excel program. This program grouped compounds into 6 categories: positive (test strain), positive (control strain), no effect, toxic/nonspecific, quencher, or unknown based on preset cutoff values. Compound data from the single-dose experiments were normalized and plotted. Compounds that were able to affect the test protein-protein interaction will have a substantially greater luminescence difference between test and control strain and were considered positive interacting compounds.

Compounds that were able to affect the interaction between the GαZ(Q205L) and RGS-Z1 fusion proteins would be identified by a decrease in *Renilla* luciferase activity (Fig. 3). Due to the multiplex format, the same compounds were simultaneously tested on the Kv4.3/KChIP (control) strain to enable determination of compound specificity. If the Kv4.3/KChIP interaction is not affected by the same compound, the YTH interaction will result in an increase of firefly luciferase activity. Nonspecific or toxic compounds affecting the GαZ(Q205L)/RGS-Z1 interaction may also affect the interaction between Kv4.3 and KChIP and would therefore result in a decrease in both the firefly and *Renilla* luciferase signals.

HTS screening and data analysis

Approximately 360,000 chemically diverse and random compounds were screened in the multiplexed dual luciferase YTH assay. *Renilla* luciferase and firefly luciferase raw data from the test and control strains were subjected to regression analysis (SAS[29] based; Fig. 4) to detect and correct automation-induced mechanical bias in the data set. Means and standard errors were calculated for the test strain and control strain data and plotted by row and column (Fig. 4A, B, C). As is evident from all plots, the samples in the outer wells of the plate exhibited a different pattern from other wells in the plate. Edge effects may be due to volume, temperature, and evaporation differences. Thus, data points from the outer wells (18%) were not included in the data analysis because they contributed significantly to mechanical bias that affects type I and type II error. Analysis of the test strain data (RGS-Z1 with *Renilla* reporter) by row enabled detection of a different plate bias; the upper left corner wells had statistically lower values than the lower right corner wells within a plate (Fig. 4A). Due to the short half-life of the *Renilla* luciferase signal, each assay well individually received *Renilla* substrate, and luminescence was determined immediately. Therefore, because the first substrate (firefly) permeabilized the cells, wells that were read late in the assay have a longer exposure, which accounts for the drift in the baseline. Linear regressions were applied to the remaining test and control strain data. The (test and control strain) data analyzed by column showed a typical up-and-down movement (Fig. 4C). This effect was present in both sets of data (test or control strain), despite the 2 luciferase

luminescences being detected using different readers, and it is most likely due to the initial fill pattern of the 384-well plates. The regression analysis, however, accounted for odd or even column placement and was able to significantly reduce the impact of this effect on subsequent data analysis.

Following regression analysis, which corrected mechanical anomalies, residual values for each compound were calculated by subtracting the plate's average from the adjusted (bias-corrected) values. Residual values for test and control strain, respectively, were plotted and were then used as a visual assessment tool (Fig. 4D). Compounds with a residual value of approximately 0 were considered to have no effect on the YTH interactions (test or control), whereas compounds with very negative residual values for both test and control strains were considered toxic or nonspecific. Preliminary testing of a subset of compounds revealed that certain compounds had a direct or indirect inhibitory effect on the luciferase enzyme or light production. These quenching compounds were found to have a small negative residual value for the test strain and a value close to 0 for the control strain and vice versa. By focusing on compounds with moderate negative residual values for the test strain versus the control strain (and vice versa), approximately 3000 compounds were selected for further analysis. After further evaluation considering Lipinkski rules[30] and additional filtering criteria,[31] 850 compounds were subselected.

HTS follow-up assays

The 850 selected compounds were tested in a single-dose assay (30 μM compound) in 2 configurations: First, the compounds were tested in the assay as described above (test strain with *Renilla* reporter gene and control strain with firefly reporter). Second, the compounds were also tested in a switched YTH assay format, where the reporter genes were paired with the other protein interaction (test strain with firefly reporter and control strain with *Renilla* reporter). The switching of reporter genes allowed determination of those compounds that have a direct (inhibiting) effect on the luminescence (false positives). The single-dose assay resulted in 4 data sets that were analyzed by a second SAS-based[29] Excel program. This program groups compounds into 6 categories based on preset cutoff values: positive (test strain), positive (control strain), no effect, toxic/nonspecific, quencher, or unknown (unclear data). Compound data from the single-dose experiments were normalized by the plate average and plotted (Fig. 4E). Compounds that affected the test protein-protein interaction have a substantially greater luminescence difference between test and control strains and are considered to be positive interacting compounds. The single-dose assay identified approximately 75 positive interacting compounds for the test strain protein-protein interaction and 65 positive interacting compounds for the control strain interaction, corresponding to a combined confirmation rate of 16.5% (140/850). This represents a more stringent validation because the follow-up assay (in contrast to the HTS assay) includes selectivity filters for toxic, quenching, and nonspecific compounds.

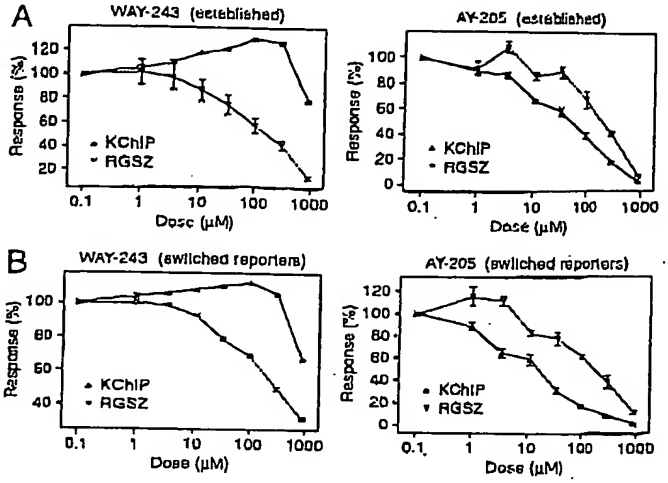

FIG. 5. Dose-response plots for 2 potential interacting compounds for test (WAY-243) and control strain (AY-205). Compounds were tested at doses between 0 and 1000 μM. The compounds were simultaneously tested in the established assay (A) and a parallel assay with switched luciferase reporter genes (B). Compounds were considered interacting when they exhibited a significant ($p < 0.001$) dose-response difference (between test and control group) over at least 3 consecutive doses and if their dose-response plots depicted similar trends when the luciferase reporter genes were switched. Data are shown as means ± SE of triplicate measurements.

The positive interacting compounds were then subsequently tested in parallel dose-response assays using established and switched reporter gene formats described above. Compounds were tested in triplicate at doses between 0 and 1000 μM. Dose-response data were then analyzed using a 2-way layout ANOVA with 3 factors: group, dose, and Group × Dose interaction (see experimental protocol). Using the Sidak-Holm step-down method,[29] compounds were considered confirmed when a significant ($p < 0.001$) dose-response difference (between test and control group) was observed over at least 3 consecutive doses and when the dose-response curves showed similar trends in both the established and switched reporter gene formats (Fig. 5). Dose-response data analysis of this compound set resulted in the identification of 7 confirmed compounds (from 75) that could inhibit the interaction between GαZ(Q205L) and RGS-Z1 (9.2% hit rate) as well as 2 confirmed compounds (from 65) that are potential inhibitors for the Kv4.3/KChIP1 interaction (3.1% hit rate). As is shown in Figure 5, WAY-243 was the most potent positive interacting compound for RGS-Z1/GαZ(Q205L) ($p < 0.001$ for all doses greater than 10 μM), whereas AY-205 was the most potent positive interacting compound for the Kv4.3/KChIP1 interaction ($p < 0.001$ for doses between 3 and 300 μM) in the dual luciferase YTH dose response analysis.

The 7 confirmed inhibitors of the RGS-Z1/GαZ(Q205L) interaction were taken forward into a single turnover GTPase assay.[15]

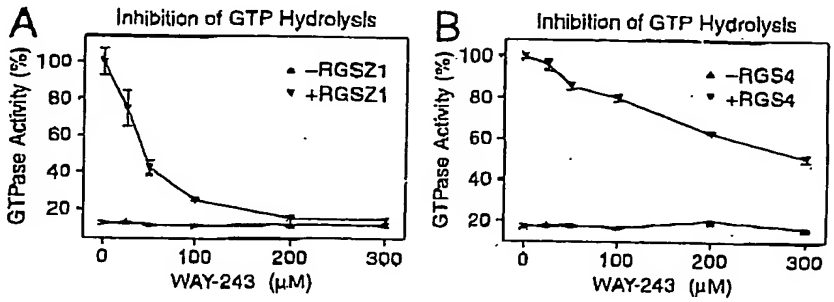

FIG. 6. WAY-243 inhibits the GTP hydrolysis of GαZ in a GTPase assay. Increasing amount of WAY-243 was incubated with either (A) 40 nM RGS-Z1 and 15 nM GαZ at 4 °C and reactions were stopped after 10 min, or (B) with 50 nM RGS4 and 50 nM Gαi-1 at 0 °C and reactions were stopped after 30 sec. Dose-dependent inhibition of GTP hydrolysis in the presence of WAY-243 is depicted as percent GTPase activity (ratio of GTPase activity [cpm] with and without RGS). Data are shown as means ± SE of triplicate measurements.

Using full-length recombinant RGS-Z1 and GαZ proteins,[15] the ability of the compounds to functionally inhibit the GAP function of RGS-Z1 was tested. Two compounds, by inhibiting the GAP activity of RGS-Z1, were able to functionally affect the GTPase activity of GαZ in a dose-dependent manner. The compound WAY-243 showed the best activity consistent with the inhibitory effects seen in yeast. WAY-243 decreased GTP hydrolysis with increasing concentrations of WAY-243 only in the presence of RGS-Z1 (Fig. 6A). The (estimated) IC_{50} of WAY-243 is approximately 50 μM. Furthermore, the selectivity of WAY-243 for inhibition of RGS-Z1 to GAP GαZ was determined by testing the compounds' ability to affect RGS4 GAP activity of Gαi-1 (Fig. 6B). GTPase results indicate that WAY-243 has only minimal effect on RGS4 GAP function, with a predicted IC_{50} of >300 μM.

DISCUSSION

Here we describe the successful adaptation of a liquid-based dual luciferase YTH assay into an HTS platform that enabled the simultaneous identification of small molecule modulators for 2 independent protein-protein interactions. The simultaneous screening of 2 independent protein-protein interactions adds speed and simplicity, as well as intra-assay well control. Initial HTS of diverse chemical entities using this dual luciferase YTH assay format resulted in more than 700,000 data points for the GαZ(Q205L)/RGS-Z1 (test strain) and Kv4.3/KChIP1 (control strain) interactions. During validation of this large data set, regression analysis of HTS data revealed a variety of automation-induced mechanical biases. An "edge effect" was detected in samples in the outer wells of the plate that exhibited a different luminescence pattern. This edge effect, which may be due to volume, temperature, and evaporation differences, contributed significantly to the mechanical bias, and therefore samples from the outer wells were eliminated from further analysis. The remaining test and control strain data contained some mechanical bias that was easily corrected by linear regressions. This first-pass regression analysis of HTS data identified approximately 3000 compounds that were considered for follow-up screening. This set of 3000 compounds was then condensed further to approximately 850 compounds using filters for drug-like properties of the representing structures. Testing this set of 850 compounds in a single-dose format identified a smaller number of compounds that could be considered as putative inhibitors for either test or control protein-protein interactions. Dose-response characteristics of these compounds resulted in the identification of 7 compounds as modulators for the GαZ(Q205L)/RGS-Z1 interaction and 2 compounds that affected the Kv4.3/KChIP1 interaction.

A small percentage of compounds was found to have direct inhibition of either firefly or *Renilla* luciferase activity or both. Quenching compounds were efficiently identified in the single-dose assay using both the established and the switched luciferase reporters. Chemical entities having quenching characteristics could potentially be false negatives, but their impact was small in relation to the successful identification of true interacting compounds. The interacting compounds identified in this study provide a base for analog and substructure searches and would therefore compensate for potential chemical entities missed in the primary HTS screen due to the mechanics of the assay format. Because the blocking of protein-protein interactions described in this study resulted in decreased luciferase activity, a higher false positive rate may be anticipated because toxic or other effects independent of the protein-protein interaction would affect reporter gene activity. The SAS-based algorithms described here enabled rapid categorization of compounds into distinct subclasses, thereby segregating true positive compounds from compounds with effects independent of the protein-protein interaction (such as toxic and luciferase quenching compounds).

One of the potential interacting compounds of the GαZ/RGS-Z1 interaction (WAY-243) was able to functionally block the GAP activity of RGS-Z1 in a single turnover GTPase assay with an IC_{50} of 50 μM (estimated). Moreover, this compound was selective for RGS-Z1 and did not have any effect on RGS4 GAP activity (IC_{50} >

300 μM). This biochemical in vitro analysis further strengthens the utility of the multiplexed dual luciferase YTH assay. Rapid identification of compounds via this platform enables SAR and combichem approaches toward development of enhanced small molecule modulators as better tools.

The compounds we have identified will be characterized in more detail in additional downstream functional in vitro and in vivo assays, such as a GTPase assay for modulators of the GαZ/RGS-Z1 interaction and electrophysiological assays for blockers of the Kv4.3/KChIP1 interaction. The multiplexed luciferase-based YTH assay accelerates the early stages of the drug discovery process by identifying new classes of pharmacological entities for 2 independent target protein-protein interactions in a relatively short time frame. These compound classes can then be subjected to further investigation in subsequent functional assay and molecular modeling efforts.

CONCLUSIONS

The multiplexed dual luciferase-based YTH assay format provides a platform amenable to high-throughput automated application adding speed and efficiency necessary for the demands and challenges associated with identifying small molecule modulators of protein-protein interactions. The Gal4 responsive luciferase reporters significantly decrease assay time, from days to hours, as compared to the YTH auxotrophic reporter systems. Furthermore, the quantitative nature of the assay data enables correction for mechanical bias and, more important, electronic capture and statistical analysis. With the development of the appropriate analysis software, primary HTS data can be analyzed rapidly, and follow-up screening of compounds can be completed within shorter time frames. Furthermore, the multiplexed assay system enabled simultaneous and efficient investigation of small molecule modulators for multiple independent protein-protein interactions. These attributes will substantially affect the identification of new drug entities, thus expanding the realm of drugable targets in the pursuit of novel therapeutics.

ACKNOWLEDGMENTS

We thank J. Cao for construction of the YTH Gal4 firefly luciferase reporter plasmid, D. Chen and A. Uveges for DNA sequencing support, L. Heydt for help with compound dilutions, K. Mason and G. Tawa for modeling support, and A. M. Gilbert for chemistry support. We further thank C. L. Hsiao for recombinant GαZ-6His cloning, W. T. Hum for recombinant GαZ-6His purification, and W. Edris for GST-RGS-Z1 purification.

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